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Sac1p plays a crucial role in microsomal ATP transport, which is distinct from its function in Golgi phospholipid metabolism

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Analysis of microsomal ATP transport in yeast resulted in the identification of Sac1p as an important factor in efficient ATP uptake into the endoplasmic reticulum (ER) lumen. Yet it remained unclear whether Sac1p is the authentic transporter in this reaction. Sac1p shows no homology to other known solute transporters but displays similarity to the N-terminal non-catalytic domain of a subset of inositol 5′-phosphatases. Furthermore, Sac1p was demonstrated to be involved in inositol phospholipid metabolism, an activity whose absence contributes to the bypass Sec14p phenotype in *sac1* mutants. We now show that purified recombinant Sac1p can complement ATP transport defects when reconstituted together with *sac1*Δ microsomal extracts, but is unable to catalyze ATP transport itself. In addition, we demonstrate that *sac1*Δ strains are defective in ER protein translocation and folding, which is a direct consequence of impaired ATP transport function and not related to the role of Sac1p in Golgi inositol phospholipid metabolism. These data suggest that Sac1p is an important regulator of microsomal ATP transport providing a possible link between inositol phospholipid signaling and ATP-dependent processes in the yeast ER.

Keywords: inositol/microsomal ATP transport/phospholipid metabolism/protein trafficking/Sac1p

Introduction

ATP transport into the endoplasmic reticulum (ER) is a prerequisite for multiple reactions inside the ER lumen. Many steps in ER protein processing are catalyzed by luminal ATP-dependent factors. In yeast, the post-translational transfer of secretory protein precursors can only occur if a sufficient level of ATP is present inside the ER lumen (Mayinger and Meyer, 1993; Mayinger *et al.*, 1995). The ATP dependence of protein translocation can be attributed mainly to the function of Kar2p, the yeast homolog of the mammalian BiP protein (Normington *et al.*, 1989; Rose *et al.*, 1989). This chaperone has two ATP-dependent functions in the post-translational mode of translocation. First, it mediates the transfer of bound precursors into the translocation pore via interaction with Sec63p (Lyman and Schekman, 1997). Subsequently, the transport of precursors through the translocation pore is catalyzed by Kar2p and ATP in a mechanism in which Kar2p may function as 'molecular ratchet' or as a 'molecular motor' (Schatz and Dobberstein, 1996). The hsp70 homologs Ssi1p and Cer1p also appear to play roles in post-translational translocation in yeast (Baxter *et al.*, 1996; Hamilton and Flynn, 1996). In additon, Kar2p and other ATP-dependent chaperones are crucial for the proper folding of translocated secretory proteins inside the ER lumen and ensure that only correctly folded proteins leave the ER and proceed along the secretory pathway toward their final destination within the cell (Gething and Sambrook, 1992; Simons *et al.*, 1995). Finally, the discovery of ATP-dependent factors such as Lhs1p as important components of the heat shock response in yeast stipulates the presence of sufficient levels of ATP in the ER lumen for thermotolerance (Saris *et al.*, 1997).

Identification and characterization of components that are required for efficient uptake of cytosolic ATP are therefore crucial to understand how ATP-dependent processes in the ER lumen are linked to the dynamic function of the ER. Although, a number of mammalian microsomal ATP transport systems have been discovered and analyzed, the molecular identity of these transporters or of components linked to ATP transport remained elusive until now (Clairmont *et al.*, 1992; Guillen and Hirschberg, 1995; Abeijon *et al.*, 1997). Recently, ATP transport activity from yeast microsomes was characterized *in vitro* in reconstituted proteoliposomes and it was shown that ATP uptake into yeast ER is mediated by an ATP/ADP antiport system (Mayinger *et al.*, 1995). Furthermore, the partial purification of transport activity led to the identification of the ER and Golgi membrane protein Sac1p as a crucial component in ATP transport. Microsomal membranes isolated from strains harboring a deletion in the *SAC1* gene (*sac1*Δ) were found to be deficient in ATP transport activity. Only low levels of ATP transport could be recovered when detergent extracts obtained from the microsomal membranes of a *sac1*Δ strain were reconstituted in the *in vitro* assay, while overexpression of Sac1p caused an increased capacity for microsomal ATP transport (Mayinger *et al.*, 1995). These findings would have been consistent with Sac1p representing the authentic ATP transporter.

The identification of Sac1p as a component in microsomal ATP transport was, however, surprising for two reasons. First, the *SAC1* gene already had been implicated in two other cellular processes by genetic analysis. Loss of the *SAC1* gene leads to suppression of certain *act1* mutant alleles (Novick *et al.*, 1989) and bypasses the requirement for the phosphatidylinositol transfer protein Sec14p for cell viability (Cleves *et al.*, 1989). *sac1* mutants also display alterations in cell wall structure and lose

structural integrity at alkaline pH (Novick *et al*., 1989; Boyum and Guidotti, 1997). Secondly, the primary sequence of Sac1p shows no homology to the structure of known solute carrier proteins. Instead, a major portion of Sac1p is homologous to the N-terminal domain of the mammalian presynaptic phosphatidylinositol 5′-phosphatase synaptojanin (Majerus, 1996; McPherson *et al*., 1996). Recently, three yeast phosphatidylinositol 5′-phosphatases were identified which also contain an N-terminal Sac1p domain (Srinivasan *et al*., 1997; Stolz *et al*., 1998a,b), but neither the exact biological function of these proteins nor the role of the N-terminal Sac1p homology domain is known. In further studies, it could be demonstrated that *sac1*Δ cells and cells expressing certain mutant alleles of *SAC1*, such as *sac1-22*, display specific changes in phospholipid metabolism which are instrumental for 'bypass Sec14p' (Kearns *et al*., 1997; M.P.Rivas, B.G. Kearns, S.Guo, Z.Xie, M.C.Sekar, K.Hosaka, S.Kagiwada, J.D.York and V.A.Bankaitis, submitted).

To address the question of how Sac1p participates in microsomal ATP transport, we examined the ability of pure recombinant Sac1p to catalyze ATP uptake into proteoliposomes. We find that Sac1p itself does not function as an ATP transporter, whereas loss of the *SAC1* gene results in ATP transport defects and ER-specific defects in protein translocation and protein folding. We conclude that Sac1p, rather than being the ATP transporter itself, is a regulatory or accessory factor required for efficient ATP transport.

Results

Expression of GST–Sac1p in sac1Δ cells

To purify Sac1p to homogeneity, we inserted the *SAC1* gene into a yeast episomal (2μ) vector next to the 5′ end of glutathione *S*-transferase (GST), placing the expression of this chimeric fusion protein under control of the inducible *GAL1/10* promoter (Mitchell *et al*., 1993). Between the GST-coding region and the *SAC1* gene a linker was present containing five glycine residues and a thrombin cleavage site to allow the recovery of recombinant Sac1p without the GST tag (Figure 1A). For all subsequent experiments, we used a *sac1*Δ background with the *reg1-501* mutation, which eliminates glucose repression of *GAL* expression (Hovland *et al*., 1989). GST–Sac1p expression could, therefore, be stimulated directly by addition of 2% galactose to the growth medium. Examination of cell extracts by SDS–PAGE and Western analysis revealed the appearance of an 89 kDa band after galactose induction which was recognized by a monoclonal antibody that is specific for the Sac1p protein (Figure 1B). This molecular weight is consistent with that expected for the GST–Sac1p fusion protein. For comparison, a 67 kDa protein band is present when the *SAC1* gene is constitutively overexpressed in *sac1*Δ cells using an episomal 2μ vector with the endogenous *SAC1* promoter (Whitters *et al*., 1993) (Figure 1B). The overexpressed GST–Sac1p in *sac1*Δ cells is ~40 times more abundant compared with the levels of Sac1p in wild-type cells. Cell fractionation and analysis of the distribution of GST–Sac1p within the different fractions revealed that essentially all the GST–Sac1p is membrane bound and co-localizes with the ER marker Kar2p (data not shown).

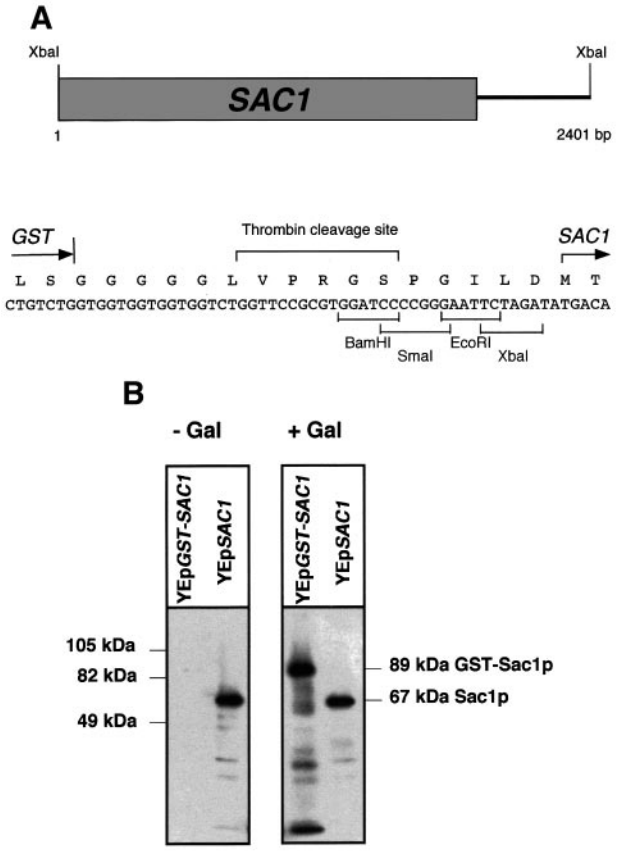

Fig. 1. Expression of a GST–Sac1p fusion protein. (**A**) Structure of the GST–*SAC1* gene fusion. The GST–*SAC1* expression vector was created by cloning an *Xba*I fragment encompassing the entire *SAC1* coding region into the *Xba*I site of pEGKT (Mitchell *et al*., 1993). In the resulting vector pPM102, the *SAC1* gene is positioned next to a linker encoding a thrombin cleavage site directly adjacent to the structural gene for GST under *GAL1/10* control (Mitchell *et al*., 1993). (**B**) Expression of GST–Sac1p in *sac1*Δ cells. Expression of the GST–Sac1p fusion protein was induced by supplementing the growth medium with 2% galactose for 7 h. Subsequently, the cells were lysed and aliquots of the extracts were analyzed by SDS–PAGE and Western blotting. As a control for the expression, extracts from cells bearing a 2μ plasmid with a genomic fragment containing the entire *SAC1* gene including its endogenous promoter were also analyzed.

Expression of GST–Sac1p complements the reduced ATP transport in sac1Δ cells

Disruption of the *SAC1* gene causes several growth phenotypes. *sac1*Δ cells are cold-sensitive, inositol auxotrophs, and the doubling time for growth in rich media is approximately twice as long as wild-type doubling times (Cleves *et al*., 1989; Whitters *et al*., 1993). To determine whether the GST–Sac1p fusion protein is functional, we tested *sac1*Δ cells containing the GST–*SAC1* plasmid for galactose-dependent complementation of these phenotypes. As shown in Figure 2A, the galactose-induced expression of GST–Sac1p confers viability to *sac1*Δ cells grown at 15°C or cultivated on inositol-free medium. In addition, the doubling time for growth of these cells on galactose-containing media is indistinguishable from that of wild-type cells (not shown). We conclude from this complementation analysis that the expressed recombinant GST–Sac1p can functionally substitute for the wild-type protein. Since it was most important to our study that the GST–Sac1p can completely fulfill the function that

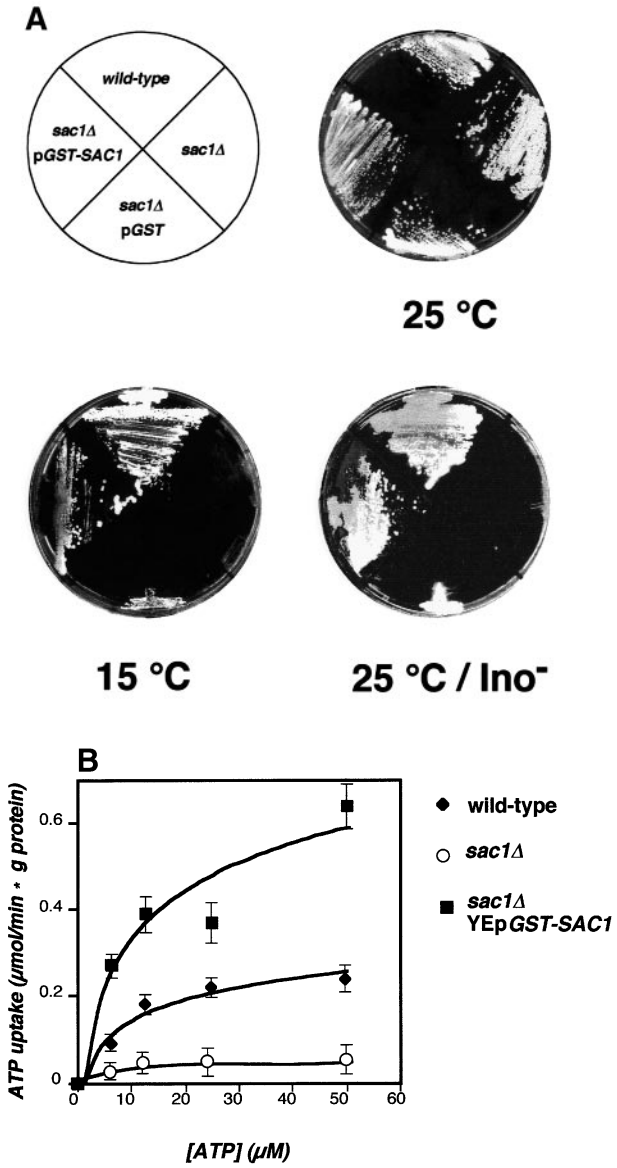

Fig. 2. Expression of a GST–Sac1p fusion protein in *sac1*Δ cells complements the specific phenotypes associated with a loss of *SAC1*. (**A**) *sac1*Δ cells containing the GST–*SAC1* fusion on a 2µ plasmid were plated on selective plates containing 2% galactose, and the complementation of the cold-sensitivity and inositol auxotrophy phenotypes was analyzed. (**B**) Microsomes from wild-type cells and from *sac1*Δ cells expressing GST–Sac1p were prepared according to Rothblatt and Meyer (1986) and assayed for specific uptake of ATP. ATP transport was measured as described in Materials and methods. Data are from three independent measurements.

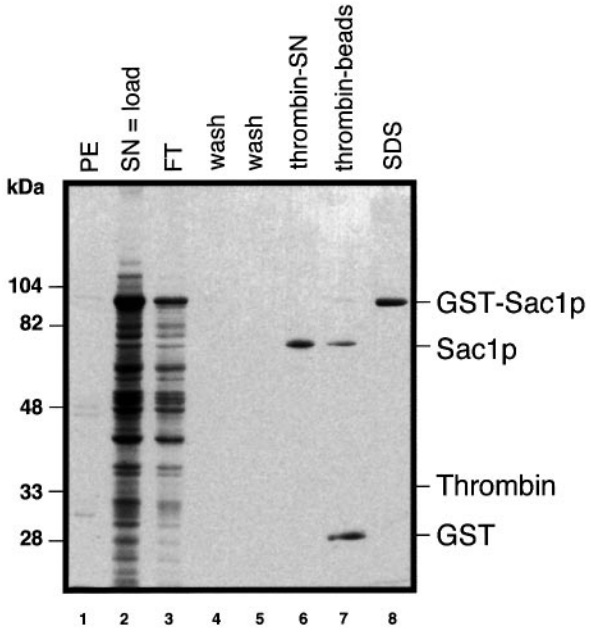

Fig. 3. Purification of recombinant Sac1p by affinity adsorption on glutathione–agarose. Microsomes containing high levels of GST–Sac1p were solubilized in 3% Triton X-100, 250 mM NaCl, 10 mM HEPES pH 7.4. The extract was centrifuged to obtain a pellet fraction (lane 1, PE) and a supernatant fraction containing the solubilized GST–Sac1p (lane 2, SN). The supernatant was diluted 1:1 with water and then mixed with glutathione–agarose beads. After 30 min incubation, the beads were washed three times with 1% Triton X-100, 125 mM NaCl, 10 mM HEPES pH 7.4 (lanes 4 and 5; only the first and the last wash fractions are shown in the figure). The beads containing the bound GST–Sac1p were resuspended in 0.5% Triton X-100, 150 mM NaCl, 2.5 mM $CaCl_2$, 50 mM Tris–HCl, pH 8.0, 0.1% β-mercaptoethanol, treated with thrombin and centrifuged to obtain a supernatant fraction containing the recombinant Sac1p (lane 6, thrombin-SN) and a pellet fraction containing the GST portion of the fusion protein which remained tightly bound to the glutathione–agarose beads (lane 7, thrombin-PE). Alternatively, an aliquot of the beads containing the bound GST–Sac1p was solubilized directly in electrophoresis buffer (lane 8, SDS).

wild-type Sac1p plays in ATP uptake, we tested microsomal ATP transport directly. Microsomal membranes from galactose-induced *sac1*Δ cells containing the GST–*SAC1* plasmid were prepared and tested for their ability to transport ATP. It was shown previously that microsomes from *sac1*Δ cells have greatly reduced ATP transport activity (Mayinger *et al*., 1995). In contrast, the GST–Sac1p-containing microsomes exhibit a four times higher capacity for ATP uptake than wild-type microsomes (Figure 2B). For comparison, the level of GST–Sac1p in these microsomes is ~40 times higher than the amount of Sac1p in wild-type microsomes (data not shown), which indicates that either some of the expressed GST–Sac1p is inactive or that the stimulating effect of GST–Sac1p in microsomal ATP transport is saturable.

Purification of recombinant Sac1p by affinity chromatography

Having ascertained that GST–Sac1p functions in microsomal ATP uptake *in vitro*, we next asked whether purified recombinant Sac1p would be sufficient for catalyzing ATP transport in reconstituted proteoliposomes. For the purification of Sac1p, we used microsomes prepared from *sac1*Δ cells expressing the GST–Sac1p fusion protein. All fractions obtained during the purification procedure were analyzed by SDS–PAGE, which is depicted in Figure 3. After solubilization with Triton X-100 and 250 mM NaCl (Figure 3, lane 2), microsomal extracts were incubated with glutathione–agarose beads. The supernatant after quick centrifugation was significantly depleted of the GST–Sac1p fusion protein (Figure 3, lane 3). This adsorption step was followed by three washes (Figure 3, lanes 4 and 5). Then, the glutathione–agarose beads containing the bound GST–Sac1p were divided into two aliquots and either treated with thrombin, to cleave off the Sac1p portion of the fusion protein (Figure 3, lane 6, thrombin-SN, and lane 7, thrombin-beads), or the bound

GST–Sac1p was eluted by boiling the beads with electrophoresis sample buffer (Figure 3, lane 8, SDS). These data show that GST–Sac1p binds specifically to glutathione–agarose beads. Thrombin cleavage of the linker between the GST protein and Sac1p results in essentially pure recombinant Sac1p that differs from wild-type Sac1p only by the presence of seven additional amino acids on its N-terminus. Using these conditions for purification, we did not observe other proteins in these fractions that could be potential interacting components of Sac1p. In rare cases, in which additional protein species were found in the thrombin-cleaved fraction, the contaminating components could be identified as breakdown products of Sac1p which were recognized by Sac1p-specific antibodies (not shown).

Reconstitution and functional characterization of purified recombinant Sac1p

To analyze the function of pure recombinant Sac1p in microsomal ATP transport, we reconstituted the purified protein into proteoliposomes and examined ATP transport activities. Two questions were addressed in these experiments. First, whether the purified recombinant Sac1p is still active. Secondly, whether the purified protein itself functions as an ATP transporter. To answer the first question, *sac1*Δ microsomes were solubilized with Triton X-100 and reconstituted with or without addition of a fraction containing pure recombinant Sac1p. In both cases, the reconstitution was performed in the presence of 10 mM ATP to obtain proteoliposomes loaded with exchangable nucleotide substrate. In previous experiments, it was shown that ATP transport into reconstituted proteoliposomes follows a nucleotide antiport mechanism and can, therefore, only be measured when ADP or ATP is present inside the liposomes (Mayinger *et al*., 1995). If extracts from *sac1*Δ microsomes were reconstituted, only a very low level of ATP uptake could be observed (Figure 4), which is consistent with our previous finding (Mayinger *et al*., 1995). However, when purified recombinant Sac1p was reconstituted together with extracts of *sac1*Δ microsomes, ATP transport activity was almost fully restored, indicating that the recombinant Sac1p is functional and sufficient to repair the ATP transport defect associated with *sac1*Δ microsomes. Next, we wanted to determine whether the purified recombinant Sac1p itself can function as a specific ATP antiporter in the reconstituted system. Accordingly, we prepared proteoliposomes containing only the purified protein. Analysis by SDS–PAGE and Western blotting confirmed that the purified recombinant Sac1p was incorporated efficiently into the phospholipid vesicles (not shown). Significantly, these proteoliposomes were completely inactive in ATP transport (Figure 4). These results provide strong evidence that Sac1p is necessary for effective ATP uptake into reconstituted proteoliposomes, yet cannot catalyze ATP transport alone.

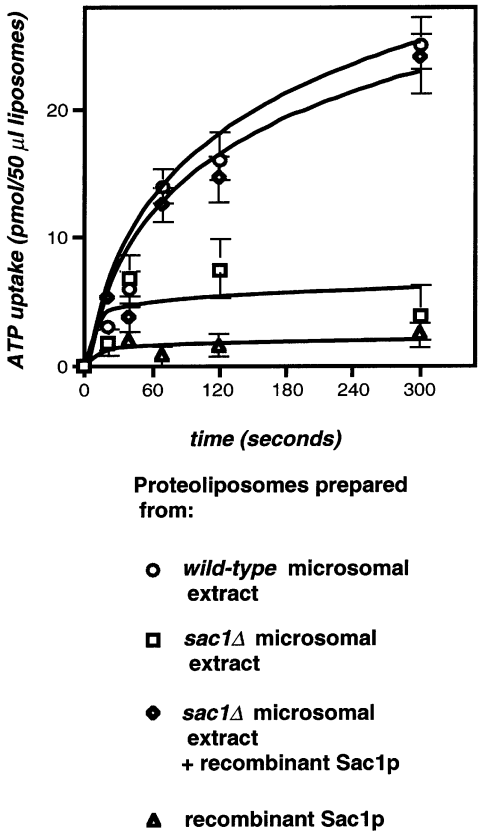

Fig. 4. Reconstitution of the recombinant Sac1p. Proteoliposomes were prepared as described in Materials and methods, using detergent extracts from wild-type microsomes or from *sac1*Δ microsomal membranes, with or without addition of 5 μg of purified recombinant Sac1p. Alternatively, proteoliposomes were prepared using only a fraction containing 5 μg of the purified recombinant Sac1p. The reconstitution mixtures were supplemented with 10 mM ATP to create liposomes loaded with exchangable nucleotide substrate. ATP uptake measurements were conducted as described in Materials and methods. Data are from three independent measurements.

The microsomal ATP transport defect in sac1Δ causes a folding defect in the ER

The collective biochemical data suggest that Sac1p is not an ATP transporter but rather an essential co-factor or regulator of microsomal ATP transport. To test whether such a function is relevant for live yeast cells, we examined how the ATP transport defect in *sac1* mutants manifests itself *in vivo*. The deficiency in microsomal ATP uptake that is seen in *sac1*Δ microsomes should lower the ATP/ADP ratio inside the ER lumen, which in turn would affect ATP-dependent steps in this compartment. We tested this by a pulse–chase analysis of ER-dependent protein trafficking. As previously observed, a complete loss of the *SAC1* gene causes a substantial defect in procarboxypeptidase Y (proCPY) transport from the ER to the Golgi complex (Mayinger *et al*., 1995) (Figure 5A). Since the function of the ER chaperone Kar2p is ATP-dependent, an *in vivo* ATP transport defect should result in increased levels of unfolded secretory proteins in the ER lumen, thus affecting the net rate of transport out of the ER. To detect such a deficiency in protein folding in *sac1*Δ cells, we examined the association of Kar2p with proCPY in the ER *in vivo* by metabolic labeling and immunoprecipitation. As shown in Figure 5A, almost no Kar2p can be co-immunoprecipitated with CPY-specific antibodies during the transit of proCPY through the ER. In *sac1*Δ cells, a substantially higher proportion of Kar2p was associated with proCPY which correlates with the accumulation of proCPY in the ER. This finding indeed points towards a malfunction of Kar2p in *sac1*Δ cells *in vivo*. These data are also in line with the previous observation that in wild-type cells only a very small portion of

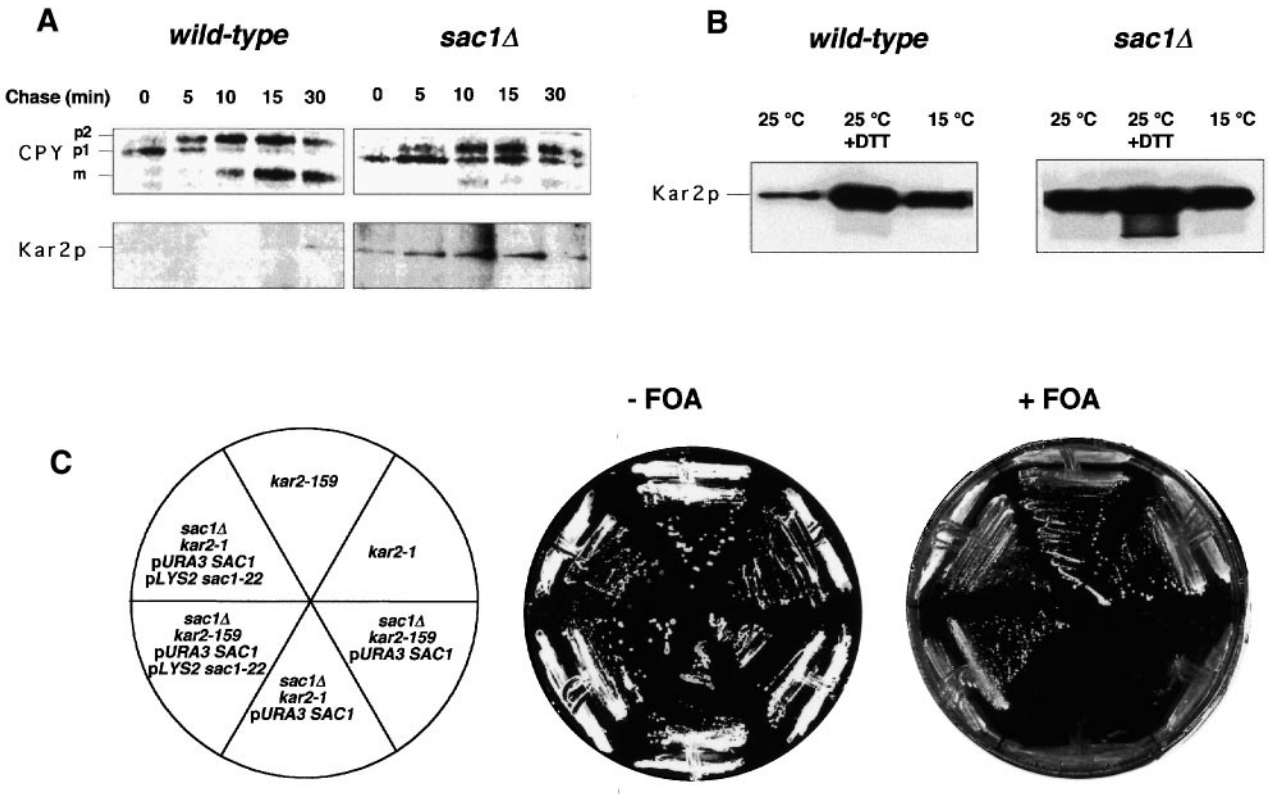

Fig. 5. The microsomal ATP transport defect in *sac1*Δ cells leads to a malfunction of Kar2p. (**A**) Cells from wild-type and *sac1*Δ cells were grown to mid-log phase. The cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 5 min at 25°C. The reactions were chased with non-labeled amino acids for the indicated times. The cells were lysed in the presence of NaN_3 and apyrase to prevent dissociation of polypeptide-bound Kar2p. The extracts were immunoprecipitated with anti-CPY polyclonal antibodies. The precipitated material was analyzed by SDS–PAGE and fluorography or by probing with anti-Kar2p antibodies to determine the portion of Kar2p associated with proCPY in the ER (p1, ER form of CPY; p2, Golgi form of CPY; m, mature CPY). (**B**) Cells from wild-type and *sac1*Δ cells were grown to mid-log phase. Three equal aliquots of each strain were incubated further for 2.5 h either at 25°C, at 25°C after addition of 10 mM DTT, or at 15°C. The cells were lysed and the extracts were analyzed by SDS–PAGE and Western blotting using anti-Kar2p polyclonal antibodies. The band below the Kar2p band, which appears in the DDT-treated samples, is a Kar2p breakdown product. (**C**) Standard genetic techniques were used to create double mutants with appropriate genotypes and containing pAT2 (*SAC1 URA3*) or pAT2 plus pPM103 (*sac1-22 LYS2*). The strains were streaked out on plates with or without FOA and incubated at 25°C for 5 days.

Kar2p can be co-immunoprecipitated with CPY-specific antibodies, while particular *kar2* mutants with a defect in the ATPase domain show significantly higher amounts of Kar2p associated with proCPY (Te Heesen and Aebi, 1994; Simons *et al.*, 1995).

The accumulation of unfolded proteins in the ER leads to activation of a signaling pathway that activates the transcription of resident ER proteins. This up-regulation of ER-resident chaperones is known as the unfolded protein response (UPR) (Cox and Walter, 1996; Shamu, 1998). Therefore, we compared the protein levels of Kar2p in wild-type and *sac1*Δ cells. As a control, the cells were incubated with the reducing agent dithiothreitol (DTT) which is known to induce the UPR (Figure 5B). Since *sac1*Δ cells cannot grow at 15°C, we also tested the influence of temperature on Kar2p levels in these cells (Figure 5B). Consistent with published results (Cox *et al.*, 1993; Kohno *et al.*, 1993), DTT induces a dramatic increase in the levels of Kar2p in wild-type cells, while a shift to 15°C leads to only slightly elevated amounts of Kar2p. In contrast, *sac1*Δ cells show a high steady-state level of Kar2p even without DTT (Figure 5B). Furthermore, addition of DTT does not lead to a further increase in Kar2p levels in these cells. These results indicate that the UPR in *sac1*Δ cells is constitutively switched on and that the level of Kar2p cannot be stimulated further by the addition of reducing agents. This finding confirms that unfolded proteins accumulate in the ER lumen of *sac1*Δ cells, which may be a consequence of reduced supply of ATP to this compartment.

We further reasoned that the intimate link between ATP transport and Kar2p function in the ER lumen would also become apparent by specific genetic interactions between *sac1* and *kar2* mutant alleles. We performed this analysis using a *sac1*Δ allele, the *sac1-22* allele, which has no deficiency in microsomal ATP transport (Kearns *et al.*, 1997), the *kar2-159^{ts}* allele, which is defective in the release of bound polypeptides, and the *kar2-1^{ts}* allele, which has a defect in polypeptide binding (Te Heesen and Aebi, 1994). To generate the appropriate genetic combinations, we transformed both *kar2* mutant strains with pAT2, a plasmid bearing a wild-type *SAC1* gene and the *URA3* auxotrophic marker, or with both pAT2 and pPM103, a plasmid containing the *sac1-22* allele and the *LYS2* marker. Then, we disrupted the genomic copy of *SAC1* in these strains and plated the cells on media with or without addition of 5′-fluoroorotic acid (FOA). Orotidine-5′-phosphate decarboxylase, the product of the *URA3* gene, catalyzes the last step in uracil biosynthesis and is essential for converting FOA to 5′-fluorouracil,

which is toxic to yeast cells (Boeke *et al*., 1987). Therefore, only cells which are viable without the pAT2 (*URA3 SAC1*) plasmid can grow on FOA-containing plates, while negative genetic interactions between the *sac1* and *kar2* alleles become visible by reduced growth on such media. As shown in Figure 5C, the *kar2-159^{ts} sac1*Δ as well as the *kar2-1^{ts} sac1*Δ double mutant strains are not viable on FOA plates at 25°C and, therefore, show a strong synthetically lethal phenotype. Importantly, the *sac1-22* allele on a plasmid is able to rescue the synthetic phenotype. The significance of this finding is discussed below.

sac1-22 strains show wild-type microsomal ATP transport and CPY trafficking

Some aspects of the pleitropic phenotype displayed by *sac1*Δ cells are based on an altered inositol phospholipid metabolism, which may complicate interpretation of our results. This aberrant inositol phospholipid metabolism in *sac1*Δ cells obviates the essential Sec14p requirement for Golgi secretory function (Cleves *et al*., 1989; Kearns *et al*., 1997; M.P.Rivas, B.G.Kearns, S.Guo, Z.Xie, M.C.Sekar, K.Hosaka, S.Kagiwada, J.D.York and V.A.Bankaitis, submitted). Consequently, one could argue that the defects in ER to Golgi trafficking, apparent in *sac1*Δ cells are an indirect consequence of alterations in inositol phospholipids, which may cause a general disturbance of intracellular membrane function. It was therefore crucial to dissect genetically and biochemically the effects that are based on an altered inositol phospholipid metabolism from the consequences of impaired ATP transport in the ER.

Recently, we identified a particular mutant allele, *sac1-22*, which displays the same aberrant Golgi inositol phospholipid metabolism as *sac1*Δ strains, but fully complements the *in vitro* ATP transport defect that is associated with a complete loss of the *SAC1* gene function (Kearns *et al*., 1997) (Figure 6A). We therefore analyzed ER to Golgi transport of proCPY in *sac1-22* cells. As depicted in Figure 6B, ER to Golgi trafficking in *sac1-22* cells is indistinguishable from that in wild-type cells, which is consistent with the wild-type levels of ATP transport that can be observed in *sac1-22* microsomes (Figure 6A). This finding provides strong additional evidence that the ER-specific defects in protein trafficking in *sac1*Δ cells are a direct consequence of impaired microsomal ATP transport which causes the malfunction of luminal ATP-dependent factors and is not related to changes in Golgi inositol phospholipid metabolism. This argument is strengthened by the observation that the *sac1-22* allele is also capable of complementing the synthetically lethal phenotype of various *kar2 sac1*Δ double mutants (Figure 5C).

sac1Δ shows specific synthetic interactions with genes coding for ATP-requiring translocon components

Since *kar2* alleles and *sac1*Δ are synthetically lethal, we wanted to investigate the genetic link between ATP transport and ATP-dependent reactions in the ER in more detail. We speculated that the ATP transport deficiency in *sac1*Δ strains would also produce a synthetic phenotype when combined with mutant alleles of genes required for ATP-dependent steps in protein translocation. To test for such genetic interactions, we used plasmid pAT2 (*SAC1 URA3*) and plasmid pAT3 (*sac1-22 TRP1*). Appropriate

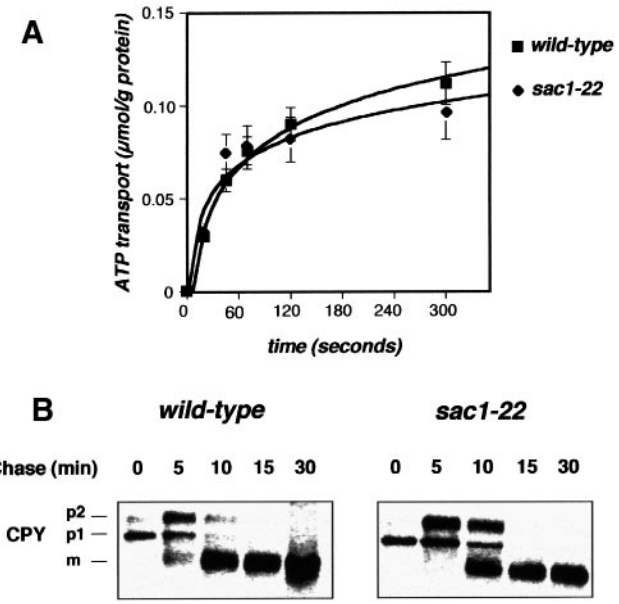

Fig. 6. The *sac1-22* mutant has normal microsomal ATP transport and CPY trafficking. (**A**) Microsomes from *sac1-22* and wild-type cells were prepared according to Rothblatt and Meyer (1986) and assayed for microsomal ATP transport as described in Materials and methods. Data are from three independent measurements. (**B**) Cells from wild-type and *sac1-22* strains were grown to mid-log phase. The cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 5 min at 25°C. The reactions were chased with non-labeled amino acids for the indicated times. The cells were lysed and the extracts were immunoprecipitated with anti-CPY polyclonal antibodies. The precipitated material was analyzed by SDS–PAGE and fluorography (p1, ER form of CPY; p2, Golgi form of CPY; m, mature CPY).

mutant strains containing the *sec61-2^{ts}*, *sec62-1^{ts}* and *sec63-1^{ts}* alleles were crossed with ATY300, a *sac1::HIS3* strain. The diploids were transformed with the plasmid pAT2 (*SAC1 URA3*) alone or with both plasmid pAT2 and plasmid pAT3 (*sac1-22 TRP1*). Tetrads from the respective transformed diploids were dissected and replica plated on rich medium and on FOA plates to select against pAT2 (*SAC1 URA3*). In cases where a significant number of spores did not grow on FOA plates, the segregation of the lethal phenotype of at least 18 tetrads was analyzed to confirm that these particular double mutants were not viable (not shown). Synthetic lethality at all temperatures was observed when the *sec61-2^{ts}* or the *sec63-1^{ts}* allele was combined with *sac1*Δ, whereas a *sec62-1^{ts} sac1*Δ strain was viable at 25 or 30°C and displayed only the ts and cs phenotype of the respective *sec62* and *sac1* alleles (Figure 7). The synthetic lethal double mutants containing pAT2 (*SAC1 URA3*) plus pAT3 (*sac1-22 TRP1*) were also analyzed on FOA plates. Importantly, all synthetic lethal combinations with *sac1*Δ were able to grow on FOA when the *sac1-22* allele was present on a plasmid (Figure 7). These results show that the synthetic interactions of *sac1*Δ with *sec61-2^{ts}* and *sec63-1^{ts}* are based on the diminished ATP transport that is associated with *sac1*Δ and are not connected with the inositol phospholipid phenotype which is present in both *sac1*Δ and *sac1-22* alleles.

Discussion

The results presented confirm the notion that Sac1p is intimately associated with microsomal ATP transport in

Fig. 7. *sac1*Δ alleles display synthetic lethality with mutations in genes coding for ATP-dependent factors in protein translocation. Standard genetic techniques were used to create strains with the appropriate genotypes containing either pAT2 (*SAC1 URA3*) or both pAT2 and pAT3 (*sac1-22 TRP1*), as indicated in the figure. The strains were streaked out on plates with or without FOA and incubated at 25°C for 5 days.

yeast. To analyze this aspect in detail, we used a GST-tagged version of Sac1p. The recombinant GST–Sac1p complemented all defects that are present in a *sac1*Δ strain. However, the biochemical data obtained with pure recombinant Sac1p do not support a hypothesis in which Sac1p represents the ATP transporter itself. The purified recombinant Sac1p was unable to confer ATP transport activity to reconstituted proteoliposomes but could complement the impaired ATP transport in reconstituted detergent extracts from *sac1*Δ microsomes when present in the reconstitution mixture. Therefore, Sac1p is likely to be an essential co-factor for efficient ATP uptake into yeast microsomes and does not represent the authentic transporter. How does Sac1p interact with the ATP transport machinery? One possibility is that Sac1p regulates ATP transport via downstream factors or by generating second messengers that bind to the ATP transporter. Phosphoinositides would be obvious candidates for such signals, though our results indicate that ATP transport is not affected by the accumulation of inositol phospholipids in membranes of *sac1* mutants. Alternatively, Sac1p may interact directly with components of the ATP transport system in the ER membrane. Our previous finding that Sac1p is a prominent constituent of a purified fraction enriched in ATP transport activity (Mayinger *et al*., 1995) is in favor of such a possibility. Additionally, it was shown before that Sac1p localizes both to Golgi and to ER membranes (Whitters *et al*., 1993), where it potentially could interact with the ATP antiport system.

There is substantial evidence that Sac1p plays an important role in regulating phospholipid metabolism. Loss of the *SAC1* gene causes inositol auxotrophy and leads to alterations in inositol phospholipid homeostasis (Whitters *et al*., 1993; Kearns *et al*., 1997; M.P.Rivas, B.G.Kearns, S.Guo, Z.Xie, M.C.Sekar, K.Hosaka, S.Kagiwada, J.D.York and V.A.Bankaitis, submitted). Aberrant phospholipid metabolism has been proposed to bypass either directly or indirectly the essential cellular requirement for *SEC14*, which encodes the yeast phosphatidylinositol transfer protein (Bankaitis *et al*., 1990; Cleves *et al*., 1991a; Kearns *et al*., 1997; M.P.Rivas, B.G.Kearns, S.Guo, Z.Xie, M.C.Sekar, K.Hosaka, S.Kagiwada, J.D.York and V.A.Bankaitis, submitted). It was therefore an *a priori* possibility that the ATP transport defect that is associated with loss of the *SAC1* gene is an indirect consequence of this effect. Strong evidence for a direct role of Sac1p in ATP transport *in vivo* comes from the analysis of the *sac1-22* missense allele. *sac1-22* strains exhibit the same dramatic changes in Golgi inositol phospholipid metabolism that are seen in a *sac1*Δ strain, but have wild-type levels of microsomal ATP transporter activity. The use of the *sac1-22* allele enabled us to link microsomal ATP transport directly to protein translocation and folding in the ER *in vivo*. *sac1*Δ displays synthetic lethality with $sec61^{ts}$, $sec63^{ts}$ and $kar2^{ts}$ alleles, while *sac1-22* on a low copy plasmid rescued these synthetic lethal phenotypes. Importantly, a *sec62 sac1*Δ combination is viable. This finding is consistent with a mechanism of protein translocation in which Sec61p, Sec63p and Kar2p participate in a functional complex that is strictly dependent on luminal ATP when engaged in the translocation of precursor proteins across the ER membrane (Panzner *et al*., 1995; Lyman and Schekman, 1997). In contrast, Sec62p functions in a step that does not require luminal ATP. Recent data indicate that Sec62p, together with Sec71p and Sec72p, represents the binding site for precursors of secretory proteins on the ER membrane (Sanders

et al., 1992; Lyman and Schekman, 1997). This binding of secretory precursors to the ER membrane was shown previously to be independent of ATP (Sanz and Meyer, 1989).

The requirement for a critical level of luminal ATP for protein translocation and protein folding and the importance of Sac1p in maintaining such a concentration of luminal ATP is substantiated further by our *in vivo* analysis of CPY trafficking in *sac1*Δ cells. It was demonstrated previously that Kar2p is an essential factor directly linked to translocation and folding of CPY in the ER lumen (Te Heesen and Aebi, 1994; Simons *et al*., 1995). Kar2p is a member of the hsp chaperone family and binds transiently to unfolded portions of translocated proteins in an adenine nucleotide-dependent cycle. In this mechanism, ATP binding to Kar2p is needed for the release of bound substrate proteins. Accordingly, one would predict that a drop in the ADP/ATP ratio in the ER lumen should lead to an increased life time of Kar2p-containing complexes and also to a decreased level of free Kar2p in the ER lumen. Our analysis of protein trafficking in *sac1*Δ cells shows that a significant portion of proCPY remains associated with Kar2p in the ER lumen, thus slowing the exit of proCPY out of the ER. In addition, the unfolded protein response pathway (Cox and Walter, 1996; Shamu, 1998) is constitutively activated in *sac1*Δ strains. These data provide strong additional evidence that the luminal ATP/ADP ratio in *sac1*Δ cells is reduced to such a level that the function of ATP-dependent luminal chaperones becomes severely compromised.

Though it is not possible at this point to assemble all these aspects into a unifying model, the collective biochemical and genetic data presented here underscore the importance of the *SAC1* gene in microsomal ATP transport and prove that this function is distinct from the role of Sac1p in regulating Golgi phospholipid metabolism. The question of how Sac1p may function in both ATP transport and Golgi phospholipid metabolism assumes particular importance, since these results show a possible connection between metabolic energy supply in the ER and Golgi function. Since Sac1p is a constituent of both ER and Golgi membranes, this protein may be involved in compartment-specific signaling events potentially via inositol phospholipids. Generation of transport vesicles is a highly regulated process that also depends on specific changes in the membrane distribution of specific types of phospholipids (Cleves *et al*., 1991b; Alb *et al*., 1996; Ktistakis *et al*., 1996; Chen *et al*., 1997). In addition, there is increasing evidence that phosphoinositides such as PtdIns(4)P, PtdIns(3)P and PtdIns(4,5)P_2 have important roles in regulating specific intracellular membrane trafficking events (De Camilli *et al*., 1996). The function of Sac1p in modulating inositol phospholipid metabolism is counter to the role of Sec14p in vesicle budding, since Sac1p defects rescue Golgi secretory function and cell viability in Sec14p-deficient yeast. (Cleves *et al*., 1989; Whitters *et al*., 1993; Kearns *et al*., 1997; M.P.Rivas, B.G.Kearns, S.Guo, Z.Xie, M.C.Sekar, K.Hosaka, S.Kagiwada, J.D.York and V.A.Bankaitis, submitted).

Changes in flux through the secretory pathway also alter the turnover of secretory proteins in the ER and Golgi. It is known that overexpression of secretory proteins in yeast causes increased synthesis of ER-resident proteins and phospholipids, which are both regulated by UPR (Cox *et al*., 1997; Menzel *et al*., 1997). Therefore, the level of ATP needed in the ER lumen has to be adapted to the respective size of the ER, which can vary substantially (Koning *et al*., 1996; Cox *et al*., 1997; Menzel *et al*., 1997) depending on the different levels of translocating secretory proteins and folding substrates passing through the ER lumen. Sac1p may therefore be a factor involved in coordinating ER and Golgi secretory competence in response to the supply of ATP to the ER. Finally, the homology of the N-terminal portion of Sac1p to the non-catalytic domain of a number of mammalian and yeast inositol-5′-phosphatases (McPherson *et al*., 1996; Kearns *et al*., 1997; Stolz *et al*., 1998a,b) provides additional evidence that Sac1p is involved in the regulation of intracellular membrane traffic by inositol phospholipids. The identification of upstream and downstream elements that interact with Sac1p and the elucidation of the role of Sac1p in inositol phospholipid signaling will be required to define more precisely the exact function that Sac1p plays in these processes.

Materials and methods

Plasmids, strains, media and reagents

The plasmid pPM102 was created by ligating a 2.4 kb *Xba*I fragment bearing *SAC1* (Cleves *et al*., 1989) into pEGKT (*URA3, 2*μ) (Mitchell *et al*., 1993), next to the structural gene for GST, which is under control of the *GAL1/10* promoter. The plasmid pPM103 is pRS317 (*LYS2 CEN*) (Sikorski and Hieter, 1989), containing the *sac1-22* allele. The plasmid pAT2 is YCplac33 (*URA3 CEN*) (Gietz and Sugino, 1988), containing the *SAC1* gene. pAT3 is YCplac22 (*TRP1 CEN*) (Gietz and Sugino, 1988), containing the *sac1-22* allele.

The strain ATY300 (*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 met-can*^{R} *reg1-501 LEU2::GAL1-lacZ sac1::HIS3*) was generated by deleting the *SAC1* gene in wild-type strain YM1862 (*MAT***a** *ura3-52 his3-200 ade2-101, lys2-801 met-can*^{R} *reg1-501 LEU2::GAL1-lacZ*) (Hovland *et al*., 1989). For construction of double mutants with the *sac1*Δ allele, ATY300 was crossed with a *sec61-2* strain (Y0098; gift from S.Jentsch, Heidelberg, Germany), *sec62-1* strain (RSY529; gift from R.Schekman, Berkeley, CA) and *sec63-1* strain (J51-5c; gift from D.Meyer, Los Angeles, CA). The diploids were transformed with pAT2 or with both pAT2 and pAT3. Double mutant derivatives with relevant genotypes were obtained by tetrad analysis. Synthetic interactions were analyzed by plating on 5′-FOA plates. *sac1*Δ *kar2* double mutants were constructed by transforming *kar2-159* and *kar2-1* strains (YG0041, YG0044; gift from M.Aebi, Zürich, Switzerland) with pAT2 or with both pAT2 and pPM103. Subsequently, the *SAC1* gene in these strains was disrupted to generate *sac1*Δ *kar2-159* and *sac1*Δ *kar2-1* strains, each containing a wild-type copy of *SAC1* or an additional *sac1-22* copy on respective plasmids to allow plasmid shuffling.

Yeast complete media (YPD), Hartwell's complete media (HC) and yeast minimal media (SD) were prepared as described (Adams *et al*., 1997). Yeast minimal media that lack inositol were prepared according to Klig *et al*. (1985). Complementation studies, meiotic segregation analyses, plasmid shuffling and gene disruptions were performed according to standard genetic techniques (Adams *et al*., 1997). GST–agarose, protein A–Sepharose, thrombin, Sephadex G-150 and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. SM2 Bio Beads were obtained from Bio-Rad. Zymolyase was obtained from ICN. Nucleotides were obtained from Boehringer Mannheim. Radiochemicals were purchased from Amersham. Polyclonal antiserum against Kar2p was a gift from G.Brodsky (University of Pennsylvania, Pittsburg, PA).

Purification of recombinant Sac1p

The strain ATY300 was transformed with pPM102 to obtain ATY300-3. Cells were grown to mid-logarithimic phase in SD medium containing 2% glucose. Expression of GST–Sac1p was induced directly by addition of 2% galactose to the medium since the strain ATY300-3 contained the *reg1-501* mutation which abolishes glucose repression of *GAL1/10* (Hovland *et al*., 1989). The cultures were incubated for an additional

6–8 h and cells were harvested by centrifugation. Isolation of microsomal membranes was performed according to Rothblatt and Meyer (1986). The GST–Sac1p fusion protein was solubilized by incubating the membranes (1 mg/ml) for 15 min on ice in 250 µl of solubilization buffer containing 3% Triton X-100, 250 mM NaCl, 20 mM HEPES pH 7.4, 1 mM PMSF and 10 µg/ml CLAP. After clearing the detergent extract by centrifugation at 20 000 *g* for 10 min, the supernatant was diluted 1:1 with water, mixed with 200 µl of 50% glutathione–agarose suspended in 125 mM NaCl, 10 mM HEPES, pH 7.4 and incubated with gentle agitation for 30 min at 4°C. The beads were washed three times with 1 ml of 1% Triton X-100, 125 mM NaCl, 10 mM HEPES, pH 7.4. and then incubated in 0.5% Triton X-100, 150 mM NaCl, 2.5 mM $CaCl_2$, 50 mM Tris–HCl pH 8.0, 0.1% β-mercaptoethanol and 0.5 U of thrombin for 20 min at room temperature. Thrombin digestion was stopped by addition of 1 mM PMSF and the beads were removed by centrifugation. The supernatant, containing the pure recombinant Sac1p, was concentrated by centrifugal filter units (Ultrafree MC, Millipore).

Preparation of reconstituted proteoliposomes

Reconstitution of detergent extracts of microsomal membranes was carried out essentially as described in Mayinger *et al*. (1995). Phosphatidylcholine/cholesterol (9:1) liposomes were prepared by sonication of a phosphatidyl/cholesterol suspension (100 mg/ml total lipid, in 100 mM MOPS, pH 7.4) in an ice bath. For reconstitution of the recombinant Sac1p, 10 µg/ml of purified protein was supplemented with a total amount of 48 mg/ml Triton X-100. To this solution, 24 mg/ml of the lipid mixture was added in small portions. After the solution became translucent, internal substrate nucleotides were added to a final concentration of 10 mM and the mix was incubated for 30 min on ice. Proteoliposomes were generated by removal of detergent accomplished by repeated passage over Bio Beads SM2 columns (1 g of SM2 beads/24 mg of Triton X-100). The proteoliposomes were separated from non-reconstituted material by gel filtration on a Sephadex G-150 column.

ATP transport assay

ATP transport into yeast microsomes was determined as described previously (Mayinger and Meyer, 1993). ATP uptake into reconstituted proteoliposomes was determined similarly as in Mayinger *et al*. (1995). A 300 µl aliquot of liposome suspension containing 10 mM ATP as counter substrate was mixed rapidly with 50 µM [^{14}C]ATP at 25°C. The exchange was stopped at different time points by rapid passage of 50 µl aliquots over 150 mg of wet Dowex 8 (chloride form, 100–200 mesh) in small glass columns. Free nucleotides are bound rapidly by the strong anion exchange resin, while liposomes are not retained. The columns were washed once with 100 µl of 100 mM MOPS, pH 7.4. Radioactivity in the combined eluate and wash was quantified by scintillation counting. Non-specific association of ATP with proteoliposomes was determined by conducting the transport assay in the presence of 0.5% Triton X-100 and by measuring ATP uptake at 0°C. All transport assays were performed in the presence of 100 µM carboxyatractyloside, a specific inhibitor of the mitochondrial ADP/ATP carrier, to rule out any influence by mitochondrial contaminants.

Metabolic labeling

Yeast strains were grown at 30°C in minimal medium (SD) to mid-log phase. Cells at 1.25 OD_{600} were harvested, washed with SD and resuspended in 1.3 ml of SD. The cells were incubated at 25°C for 15 min and then labeled with 0.5 mCi of Trans ^{35}S-label for 5 min. Subsequently 1.3 ml of 2× chase solution was added (final concentration: 0.03% methionine, 0.03% cysteine and 0.03 M ammonium sulfate). Aliquots were removed at different time intervals and quenched on ice with 10 mM NaN_3. The samples were pelleted and washed in phosphate-buffered saline. Glass bead lysis and denaturing immunoprecipitations were conducted as described earlier (Mayinger *et al*., 1995). For native immunoprecipitations, cell pellets were resuspended in 250 µl of 2% CHAPS, 200 mM NaCl, 50 mM HEPES pH 7.4 and lysed with 200 µg of glass beads in the presence of 1 mM NaN_3 and apyrase (1 U) to prevent dissociation of polypeptide-bound Kar2p. The extracts were immunoprecipitated with anti-CPY polyclonal antibodies. The precipitated material was analyzed by SDS–PAGE and fluorography or by probing with anti-Kar2p antibodies to determine the portion of Kar2p associated with proCPY in the ER.

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